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Immunocytokines: a new appr ach to immunotherapy of melanoma

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Targeted interleukin-2 (IL-2) therapy with immunocytokines (i.e. antibody–cytokine fusion proteins) is effective in eradicating established hepatic and pulmonary metastases of melanoma in animal model systems. The effector mechanisms responsible for this antitumor effect in syngeneic, immunocompetent mice involves mainly CD8+T cells. This was clearly indicated by immunohistochemical analyses, *in vivo* depletion studies and cytotoxicity tests. Such CD8+T cells, isolated from tumor-bearing mice after immunocytokine therapy, exerted a major histocompatibility complex class I-restricted cytotoxicity against the same tumor *in vitro*. Because of this cellular immune response, antibody-directed IL-2 therapy can even address established metastases displaying extensive heterogeneity in the expression of the targeted antigen. The effector mechanisms induced by immunocytokines facilitate partial regressions of large subcutaneous melanoma exceeding more than 5% of the body weight. These results demonstrate the ability of immunocytokines to induce a T-cell-dependent host immune response capable of eradicating established melanoma metastases in clinically relevant organs and offers an effective, new tool for immunotherapy of malignant melanoma.

Keywords: Immunocytokines, targeted interleukin-2 therapy, melanoma.

Introduction

One of the major goals of melanoma immunotherapy is the induction of tumor-specific T-cell responses that will be effective in the eradication of disseminated tumors. The rationale for this approach is based on the fact that melanoma cells are antigenic, because they express tumor-associated antigens consisting of peptides that are recognized by syngeneic T cells. However, most progressively growing neoplasms, such as melanoma, fail to provoke antitumor immune responses that are capable of controlling the growth of malignant cells. Almost two decades ago, Talmage *et al.* [1] described a tumor cell line that could not induce an allogeneic T-cell response despite its apparently normal expression of major histocompatibility complex (MHC) molecules. However, these investigators demonstrated that this defective T-cell activation could be restored by cytokines [1], thereby providing the rationale for cytokine-based forms of tumor immunotherapy.

A number of clinical trials have been based on the systemic administration of immunomodulatory cytokines, such as interleukin-2 (IL-2), one of the most potent antitumor cytokines [2,3]. Thus, IL-2 exhibits a wide variety of biological activities, including the stimulation of proliferation of antitumor effector cells, such as cytotoxic T cells (CTL), T-helper cells and natural killer (NK) cells [4]. However, the systemic administration of IL-2 failed to recognize its paracrine mode of action that requires concentration in the tumor microenvironment [5]. Instead, systemic administration of IL-2 and other cytokines in pharmacological doses produced high concentration of these molecules in the vasculature at sites distant from the tumor, but often at suboptimal levels in the tumor microenvironment [3]. Furthermore, such pharmacological doses of IL-2 often result in potentially life-threatening side-effects [4].

A therapeutic approach that recognizes the paracrine mode of action of cytokines that function optimally within a few cell diameters of their cell of origin, is the *ex vivo* genetic modification of tumor cells to express cytokines *in situ* [6]. Such cytokines produced by transducing genes encoding them into autologous tumor cells induce a local inflammatory response resulting in the elimination of injected tumor cells. In some cases, systemic immune responses are generated against challenge with the wild-type parental tumor [7]. Although highly encouraging preclinical data were obtained with this gene therapy approach, its broad application as a cancer therapy appears limited by its patient-specific nature.

We recently developed an alternative approach for cancer therapy that combines effective local concentrations of cytokines in the tumor microenvironment, low systemic toxicity and simple *modus operandi*. Importantly, this application is not limited by being patient-specific. We achieved this by construction of recombinant fusion proteins consisting of tumor-spe-

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cific monoclonal antibodies (MAb) and cytokines [8,9]. We named these antibody–cytokine fusion proteins 'immunocytokines', since they employ the unique targeting ability of antibodies to direct multifunctional cytokines to the tumor site and induce tumor-specific cellular immune responses that will eradicate the tumor.

This overview summarizes some of our results obtained in a series of studies that evaluated the efficacy of immunocytokines in eradicating established melanoma metastases in animal models.

Construction and *in vitro* characterization of immunocytokines

Construction

Gillies *et al.* [10] reported on a recombinant fusion protein consisting of chimeric anti-ganglioside GD_2 antibody (ch14.18) and recombinant human IL-2 (rhIL-2) designated ch14.18–IL-2. In constructing this immunocytokine, plasmid constructs were used where the immunoglobulin–IL-2 fusion protein expression vector was constructed by fusing a synthetic human IL-2 sequence to the carboxyl end of the human Cγ1 gene. A synthetic DNA linker was employed to join the NH_2-terminal codon of mature IL-2 to the exact end of the H-chain gene (ch14.18–IL-2). The fused gene was inserted into the vector pdHL2-14.18, as described previously, for an antibody–lymphotoxin fusion protein [8]. The expression plasmid was introduced into the immunoglobulin-nonproducer murine hybridoma cell line, Sp2/0-Ag14 by protoplast fusion and selected in medium containing 100 nM methotrexate. The clones secreting the largest amount of ch14.18–IL-2 were propagated in increasing concentrations of methotrexate and subcloned in medium containing 5 μM methotrexate. Purification of the ch14.18–IL-2 fusion protein was performed on protein-A Sepharose [8,9].

Characterization of immunocytokine functions

Construction of an antibody–IL-2 fusion protein by fusing the coding sequence of IL-2 to the end of the H-chain gene resulted in a fully assembled antibody fusion protein (ch14.18–IL-2) with full IL-2 activity. Interestingly, this construct had enhanced antigen-binding activity when compared with MAb ch14.18, although this effect has not been observed with other antibody IL-2 fusion proteins. Evaluation of biological activities of ch14.18–IL-2 indicated that fusion of IL-2 to the carboxyl terminal of the immunoglobulin H-chain did not reduce IL-2 activity when measured in proliferation assays with either mouse or human T-cell lines. Also, ch14.18–IL-2 was remarkably stable throughout its purification and during subsequent storage for up to 4 years, thus far, at −20°C. The effector functions of this immunocytokine, i.e. the ability to mediate complement- or antibody-dependent cellular cytotoxicity, were found to be maintained, although somewhat decreased when compared with MAb ch14.18.

The ch14.18–IL-2 fusion protein exhibited enhanced tumor infiltrating lymphocyte (TIL) cytotoxic activity of autologous tumor targets. This was observed when the human 660 TIL line, which is CD3+, CD8+, antigen-specific and MHC class I-restricted, was used to test the ability of the ch14.18–IL-2 fusion protein to stimulate the killing of GD_2-positive autologous melanoma tumor cells (660mel). In fact, the ability of testing 660 TIL cells was enhanced if the melanoma target cells were first coated with the immunocytokine. This stimulation of tumor cell killing was greater than that of uncoated tumor cells in the presence of equivalent or higher concentrations of free IL-2 [10].

An additional series of *in vitro* experiments were performed in an attempt to gain an understanding of some of the mechanisms by which cellular cytotoxicity is mediated by a recombinant antibody–IL-2 fusion protein directed against human melanoma cells. In this case, functional characteristics were established for the fusion protein between rhIL-2 and the mouse–human chimeric MAb 225 (ch225), directed against the human epidermal growth factor receptor (EGFR). Antigen-binding activity of ch225–IL-2 was essentially the same as that of MAb ch225. This was evident from dissociation constants (K_D) for radiolabeled ch225 (2.62×10^{-10} M) and ch225-IL-2 (3.31×10^{-10} M) calculated from saturation binding curves obtained with the human melanoma cell line M24met. The biological activity, in terms of IL-2-induced proliferation of the IL-2-dependent cell line CTTL-2, was the same as that of equivalent amounts of rhIL-2 when calculated as two molar equivalents of IL-2 per mole of ch225–IL-2 [11]. The amount of cytotoxicity mediated by fresh human peripheral blood mononuclear cells against human melanoma 24met and C8161 target cells coated with either ch225 or ch225–IL-2 indicated that the immunocytokine was superior to ch225 in this regard. As far as different effector cell populations are concerned, ch225 and ch225–IL-2-mediated NK-cell cytotoxicity, while resting T cells were not stimulated by either ch225 or ch225–IL-2. However, unlike fresh peripheral blood mononuclear cells, purified NK cells coated with ch225 or ch225–IL-2 killed both melanoma target cells with the same efficacy [11].

In experiments designed to assess the relative involvement of Fcγ receptor (FcγR)-III and IL-2 receptor in NK cell-mediated cytotoxicity, NK cells interacted with ch225–IL-2 mainly through FcγRIII, while the involvement of IL-2 receptor was secondary. When human T cells were activated through CD3 and stimulated with exogenous IL-2, they were found capable of killing melanoma cells, an effect further enhanced by the presence of ch225–IL-2. Since CD8+ cell populations were much more potent effectors than CD4+ T cells, we tested the cytotoxicity of activated CD8+ T cells mediated by ch225–IL-2 against human melanoma cells. In this case, an equivalent amount of rhIL-2 was also found capable of stimulating activated T cells to lyse melanoma cells. These data imply that the effect of

ch225–IL-2 on activated T cells was most likely mediated through IL-2 receptor. This is in contrast to observations made with NK cells, where as little as 0.1 μg/ml ch225–IL-2 was effective in stimulating these effector cells, while equivalent amounts of ch225–IL-2 or rhIL-2 did not potentiate T cells [11].

Taken together, all these *in vitro* data suggest that ch14.18–IL-2 and ch225–IL-2 may be potent immunotherapeutic agents that are able to recruit effector cells expressing FcγRs and that they may also be capable of delivering relatively large amounts of IL-2 to the tumor microenvironment to locally activate suitable effector cells. These data encouraged us to critically evaluate the antitumor activity of these immunocytokines in a series of animal models of metastatic human and murine melanoma.

Eradication of human hepatic and pulmonary melanoma metastases in severe combined immunodeficient mice by immunocytokines

In an effort to critically evaluate the effect of these immunocytokines on melanoma metastases *in vivo*, we employed a number of different experimental models using tumor cells expressing either EGFR or the ganglioside GD_2 [12]. In a first set of experiments, we addressed the effect of fusion proteins on the dissemination and growth of hepatic metastases. The experiment model consisted of EGFR-expressing human melanoma cells (M24met) that form hepatic metastases after intrasplenic injection of 5×10^5 cells into C.B-17 *scid/scid* mice when followed, within 60 s, by ligation of the splenic pedicle. Treatment of these animals 24 h after tumor cell inoculation by intraperitoneal reconstitution with 10^7 human lymphokine-activated killer (LAK) cells followed by intraperitoneal injection of 16 μg ch225–IL-2 fusion protein on seven consecutive days, completely suppressed the growth of hepatic tumors in all animals. This complete suppression of tumor growth was confirmed by histologic examination of consecutive sections of liver specimens. In contrast, neither the treatment of animals by reconstitution with LAK cells alone nor with a combination of ch225 plus equimolar concentration of recombinant IL-2 (16 μg ch225 plus 48 000 IU IL-2) significantly suppressed the outgrowth of hepatic metastases.

Although we could not detect metastatic foci by macroscopic/histologic examination in the animals following treatment with the tumor-specific antibody-IL-2 fusion protein plus LAK cells, it is still possible that the mice could suffer from minimal residual disease. We ruled out this possibility by the use of two different strategies. First, the detection of human melanoma cells at the molecular level by reverse transcriptase polymerase chain reaction (RT-PCR), and second, an evaluation of the effect of this treatment on animal survival time. In our hands, the sensitivity of tracing human M24met tumor cells by detecting mRNA encoding human tyrosinase by RT-PCR was approximately 10 cells among 10^6 murine cells. When tumor-bearing mice were treated with the combination of ch225 and recombinant IL-2, M24met cells were detected by RT-PCR in the liver during the whole experimental period of 3 weeks. In contrast, in livers of animals treated with the tumor-specific ch225–IL-2 fusion protein, human tyrosinase messenger RNA was only detected until day 8, indicating the eradication of tumor cells by that time [12].

A series of survival studies performed to measure the extent of residual disease after treatment with the antibody–IL-2 fusion protein indicated a mean survival time of 45.4 days for C.B-17 *scid/scid* mice after induction of experimental hepatic melanoma metastases and reconstitution with LAK cells. This survival time was not significantly altered by the administration of ch225 plus rIL-2 (mean 47.0 days); however, it was more than doubled by treatment with the ch225–IL-2 fusion protein. When the experiment was terminated after 98 days, the remaining animals still showed no overt signs of any malignant disease. Post mortem examination indicated that one animal, that died on day 88, had a brain metastasis that obstructed the circulation of the cerebrospinal fluid and caused a severe hydrocephalus internus. In the group of seven animals sacrificed on day 98, a second case of grossly visible brain metastases was observed.

Since another major site for distant metastases of melanoma is lung, we tested the hypothesis that lung involvement of metastatic melanoma could also be successfully treated by antibody–IL-2 fusion proteins. To this end, we induced experimental lung metastasis in C.B-17 mice by intravenous injection of 5×10^5 M21 cells expressing ganglioside GD_2 recognized by MAb ch14.18. Similarly, as observed with the hepatic metastasis model, the tumor-specific ch14.18–IL-2 fusion protein completely inhibited the outgrowth of any tumor metastases when given 24 h after tumor cells injection. Neither LAK cells alone nor in combination with recombinant IL-2 plus ch14.18 were able to achieve the same tumor-suppressive effect [12].

We also addressed the cruial issue whether immunocytokines can effect the growth of established tumors by delaying the start of ch14.18–IL-2 administration to assess the timepoint at which an effective treatment can still be achieved. We found that the complete eradication of hepatic tumor metastasis could be demonstrated in five out of six animals and for pulmonary metastases, in four out of six animals, if treatment with ch14.18–IL-2 is started on day 8, after inoculation of tumor cells. Established and disseminated micrometastases of M21 human melanoma cells in lung and liver tissues were clearly detectable by histologic examination at this time point. Even a delay of therapy, until day 12, still resulted in a substantial reduction in tumor load compared with untreated animals; however, none of these animals could be cured [12].

Another issue to be addressed is whether the altered pharmacokinetic property of ch14.18–IL-2 ($t_{1/2\alpha}$ 0.3 h; $t_{1/2\beta}$ 30 h), compared with the very brief half-life of IL-2 ($t_{1/2\beta}$ 6 min) is responsible for the therapeutic effect observed with ch14.18–IL-2. To this end, C.B-17 *scid/scid* mice injected with M24met melanoma cells, reconstituted with human LAK cells, were

treated with the combination of ch225 and Fc–IL-2 fusion protein. The latter protein has pharmacokinetics similar to the ch225–IL-2 fusion protein, but lacks the Fab fragment of an intact antibody, along with antigen binding activity. Although this treatment proved to be superior to the combined administration of ch225 and IL-2, it only led to a reduction in tumor load compared with the complete suppression of tumor growth by treatment with the ch225–IL-2 fusion protein [12].

We further proved the specificity of the tumor-suppressive effect of the antibody-IL-2 fusion proteins by using the melanoma cell line M21 to induce hepatic metastasis in C.B-17 *scid/scid* mice. This cell line expresses high levels of ganglioside GD_2, but lacks expression of the EGFR recognized by MAb ch225 [13]. As in the previous experiments, treatment was started 24 h after intrasplenic injection of tumor cells and consisted of reconstitution with human LAK cells alone or in combination with the fusion proteins. However, in this experiment, we used either the tumor-specific fusion protein, ch14.18–IL-2 or one not reacting with the tumor, i.e. ch225–IL-2. Only the tumor-specific fusion protein completely suppressed the growth of tumor metastasis, whereas the nonspecific fusion protein exerted a similar effect on tumor growth as Fc–IL-2.

T-cell-mediated eradication of murine metastatic melanoma induced by immunocytokines

As described above, we demonstrated in a series of metastatic models in immunodeficient mice that immunocytokines effectively combine the unique targeting ability of antibodies with the multifunctional activity of cytokines to eradicate human hepatic and pulmonary melanoma metastases [14,15]. Since all these tumor models in immunodeficient mice lack mature T cells, we tested the hypothesis that immunocytokines can effectively induce tumor-specific T-cell responses that will lead to the eradication of disseminated melanoma metastases. These experiments were performed in a fully immunocompetent syngeneic murine melanoma model that enabled us to analyze effector mechanisms responsible for this therapy-induced tumor eradication.

Specifically, to test the effect of these antibody–IL-2 fusion proteins on distant melanoma metastases *in vivo*, a number of different experimental tumor models were employed using B16 melanoma cells, which were transfected with genes coding for β-1,4-N-acetylgalactosaminyltransferase and α-2,8-sialyltransferase resulting in a constitutive expression of the ganglioside GD_2, the antigen recognized by ch14.18–IL-2 [16]. These tumor cells formed experimental pulmonary and hepatic metastases following intravenous or intrasplenic injection, respectively. After 1 week, these were present as disseminated, established micrometastases [14,15].

The effect of the antibody–IL-2 fusion protein on disseminated, established pulmonary metastases was tested by treating mice 1 week after tumor cell inoculation with 8 μg ch14.18–IL-2 fusion protein injected intravenously for 7 days. This treatment completely eradicated pulmonary micrometastases in 14 out of 16 animals, a fact that was confirmed by histologic examination of serial sections of lung specimens. The remaining two animals' tumor loads were dramatically reduced to one or two grossly visible pulmonary foci, compared with control animals that suffered from more than 500 pulmonary metastases, after receiving either no treatment or a combination of equimolar amounts of rIL-2 and ch14.18 MAb. Similar results were obtained when animals bearing disseminated hepatic metastases established for 7 days were treated intravenously with 8 μg ch14.18–IL-2 fusion protein for 7 days. This treatment resulted in complete regression of micrometastases in seven out of eight animals. The antitumor effect of ch14.18–IL-2 fusion proteins was shown to be specific, since ch225–IL-2, a fusion protein not reactive with the tumor cells, failed to exert any antitumor effects [15].

Survival studies, performed to measure any metastases not detected by macroscopic or histologic examination, indicated a mean survival time of 41 days after induction of experimental pulmonary metastases in mice receiving no further treatment. This survival time was not significantly changed by the administration of ch14.18 antibody in combination with recombinant IL-2 (mean 44 days). However, survival time was more than doubled by treatment with the ch14.18–IL-2 fusion protein. Essentially equal results were observed in mice suffering from hepatic metastases because animals lived at least twice as long after therapy with ch14.18–IL-2 than control animals [15].

One other major site for distant metastases of human melanoma, in addition to the lung, lymph node and liver, is the skin. Therefore, we tested the effect of antibody–IL-2 fusion protein treatment on established subcutaneous tumors. Ten days after inoculation of tumor cells, animals were treated over a period of 7 days by intravenously administered ch14.18–IL-2 fusion protein (16 μg per injection). Objective responses were observed in all treated animals; three out of eight animals had complete remissions and five out of eight animals had partial remissions. Even if treatment was delayed as long as 35 days, resulting in large subcutaneous tumors (~1000 mm^3), ch14.18–IL-2 was able to induce a partial regression of the tumor and delayed its future growth. The differences in tumor weight between animals receiving the fusion protein or no treatment were statistically significant with two-tailed P values equal to or less than 0.01 [14].

Our previous observations indicated that fusion proteins localize in antigen-expressing subcutaneous tumors in nude mice. In addition, the selectivity of localization increased over time, since the fusion protein was more slowly eliminated from the tumor than from blood or other organs [12]. However, the more relevant clinical question is whether the fusion protein is able to target micrometastases in affected organs, such as lung and liver. Thus, we tested the hypothesis that the ch14.18–IL-2 fusion protein can target micrometastases in affected organs of a syngeneic host and we examined the biodistribution of ^{125}I-labeled ch14.18–IL-2 in C57BL/6J mice with either hepatic or

pulmonary metastases 10 days after their experimental induction. When the amount of radioactivity in livers and lungs of such animals were assessed 12 h after intravenous injection of the fusion protein, a strong localization of tumor-specific ch14.18–IL-2 was observed within the tumor-bearing organs [15].

One of the major obstacles thwarting antibody-based immunotherapy is the heterogeneity of antigen expression within the tumor. We previously proposed that successful treatment with antibody–IL-2 fusion proteins may be achieved with only a small percentage of the tumor mass being targeted by the fusion protein, since these targeted cells may serve to elicit a specific cellular immune response. In order to test the hypothesis that antibody–IL-2 fusion protein treatment can overcome tumor cell heterogeneity, pulmonary metastases were induced that were heterogeneic in GD_2-positive and negative B16 melanoma cells at a ratio of 5 : 1. Treatment with ch14.18–IL-2 dramatically reduced the number of metastatic foci on the lungs of five out of eight animals and induced a complete cure in three out of eight animals. If only GD_2-negative cells were used for inoculation, ch14.18–IL-2 displayed no antitumor effect, proving the specificity of this treatment [14].

Animals bearing 50 ml subcutaneous tumors induced by inoculation of GD_2-positive murine melanoma cells were treated by intravenous injection of 8 µg ch14.18–IL-2 fusion protein on seven consecutive days. When tumors and surrounding tissue were excised on days 3, 5 and 7, after initiation of therapy and histologically examined, only biopsies of tumors from animals treated with the fusion protein showed a marked inflammatory response, with infiltration of lymphocytes, macrophages and occasional neutrophils, at all examined timepoints. Staining with an antibody reacting with the CD45 antigen confirmed the presence of leukocytes within the tumor of treated mice and the absence of these cells in control animals treated with a mixture of rhIL-2 and ch14.18. Further characterization of the lymphocytic infiltrate with antibodies reacting with CD4 or CD8 identified a large portion of these as CD8+ T cells; a smaller percentage of the infiltrate could be accounted for by CD4+ cells. Neither CD8 or CD4, and only a few CD45 expressing cells, were detectable in tumors obtained from control animals. Staining of tumor specimens with NK 1.1 antibody identifying natural killer cells in C57BL/6 mice, showed only occasional cells in tissues surrounding the tumor and none infiltrating the tumor. No obvious differences in fusion protein-treated or control animals were detected in this regard [14].

The immunohistologic characterization of the inflammatory infiltrate in GD_2-positive B78-pD3T-31 tumors induced by antibody–IL-2 fusion protein treatment argues against a relevant role of NK cells in the tumor eradication. To formally prove this conclusion, established pulmonary melanoma metastases in C57BL/6 *beige/beige* mice, lacking any functional NK cells, were treated with immunocytokines, caused a complete eradication of the tumor micrometastases, indicating that NK cells are not required [15].

The relevance of T cells for the observed tumor eradication was addressed by injecting GD_2-expressing tumor cells intravenously into T-cell-deficient C57BL/6 *scid/scid* mice followed by therapy with ch14.18–IL-2 7 days thereafter. In the absence of T cells, the therapeutic efficacy of the antibody–IL-2 fusion protein is dramatically reduced, since none of the eight animals showed a complete eradication of micrometastases. This observation strongly suggests a T-cell-dependent mechanism for the demonstrated antitumor effect. Thus, the participation of T-cell subsets in the fusion protein-induced tumor regression was investigated by *in vivo* depletion of these cells with specific antibodies. Since NK cells might be able to substitute for some functions of depleted T-cell populations, these studies were performed in C57BL/6 *beige/beige* mice. Eradication of 7-day established pulmonary metastases was achieved by treatment with ch14.18–IL-2 in nondepleted and CD4+ cell-depleted mice. Depletion of CD8 or both CD4/CD8-expressing cells abrogates the therapeutic effect of the administered fusion protein. Provided that depletion of each T-cell subset was effective and specific, these results suggest that only the presence of CD8 T cells is mandatory for eradication of the metastases [15].

The third line of evidence indicating the involvement of CD8+ T cells was provided by cytotoxicity studies. To this end, spleen cells were isolated from mice after induction of pulmonary metastases and subsequent treatment with the tumor-specific antibody–IL-2 fusion protein. These cells displayed a high cytolytic activity against GD_2-expressing tumor cells in a 4 h ^{51}Cr-release assay. Enrichment for CD8+ T cells demonstrated that this cell population provides a major contribution to the observed cytolytic activity. In addition, blocking studies with antibodies directed against H-$2K^b$/H-$2D^b$ antigens proved that the killing of B78-D14 melanoma cells by either unselected primed lymphocytes or the CD8+ subset thereof is MHC class I-restricted. In contrast, spleen cells from tumor-bearing mice treated with the same amount of fusion protein (ch225–IL-2), not reacting with the tumor, showed no specific lysis of tumor cells [15]. These data, combined with results from the *in vivo* depletion studies, clearly indicate that the antitumor effect achieved by the ch14.18–IL-2 immunocytokine is specific and largely dependent on CD8+ T cells, which kill tumor cells in an MHC class I-restricted manner.

Perspectives

The data summarized in this overview demonstrate that the unique tumor targeting ability of immunocytokines renders them therapeutically effective for the treatment of established hepatic, pulmonary and subcutaneous melanoma metastases in animal model systems. Even micrometastases displaying some degree of antigenic heterogeneity could be successfully addressed by this type of therapy. Several lines of evidence, i.e.

immunohistology, *in vivo* depletion studies and cytotoxicity assays, indicated that this antitumor effect largely depends on CD8+ cells [14,15].

The idea of using the targeting ability of certain molecules to direct therapeutics to the desired microenvironment was proposed by Paul Ehrlich almost a century ago [17]. More recently, research efforts were initiated to prove the feasibility of targeting radioisotopes, cytotoxic drugs or potent toxins by conjugating them to MAb. Although some encouraging results were obtained in clinical trials applying radioimmunoconjugates for the treatment of patients with non-Hodgkin's lymphomas and myeloid leukemias, their therapeutic efficacy in solid tumors has remained very low [18–21]. Our approach is novel, since it attempts to induce a tumor-specific cellular immune response by localizing IL-2 in the tumor microenvironment. As documented in this overview, it is possible to achieve effective concentrations of IL-2 at the tumor site *in vivo* by targeting it to the tumor via the binding specificity of MAbs. Biodistribution studies with ^{125}I-labeled ch14.18–IL-2 indicated that localization of this fusion protein in subcutaneous tumors, as well as livers and lungs bearing micrometastases is sufficient to achieve effective local cytokine concentrations at the tumor site in a nonpersonalized way. In fact, multiple and conventionally inaccessible tumor sites can be targeted, which is especially important for the treatment of minimal residual disease.

Considerable knowledge about the effects of IL-2 present in the tumor microenvironment on the induction of an antitumor T-cell response has been gained through *in vivo* experiments using tumor cells genetically engineered to produce IL-2 [22–27]. Initial reports indicated that IL-2-producing tumor cells induce an antitumor MHC class I-restricted CTL response in the absence of CD4 help [22]. However, other findings demonstrated that such tumor cells can be rejected in nude mice, indicating that T cells are not critically needed [23–25]. In fact, the absence of T cells at the site of primary immunization in euthymic mice has also been demonstrated [25,26]. This is consistent with reports on the circulation patterns of naive versus memory or activated T cells indicating that naive cells, as opposed to memory or activated T cells, do not access peripheral tissue [28]. Although these observations suggest that rejection of IL-2-expressing tumor cells involves primarily T cell-independent host defense mechanisms, e.g. macrophages or granulocytes, the published studies to date clearly demonstrate that the elimination of wild-type tumor cells inoculated either before or after vaccination is critically dependent on the presence of tumor-reactive T cells [22,24,27,29,30].

The experimental setting of our studies differs from those investigating the host immune response to IL-2-producing tumor cells, since we targeted IL-2 to the microenvironment of tumors established for 7–14 days prior to the start of treatment. This different experimental setting is important, since it is of high clinical relevance, and may change immune reactions in several ways. In fact, the host immune system most likely encountered the tumor before the immune modulation was initiated. Consequently, T-cell priming could already have occurred via such antigen-presenting cells as macrophages or dendritic cells, especially since tumor cells are more sensitive to the innate cytodestructive effects of these cells immediately after inoculation than after establishment in micrometastases. It is also possible that some tumor cells did metastasize to the draining lymph nodes during this period, thereby overcoming the problem of naive T cells being compartmentalized in blood and lymphoid organs. In this regard, fibroblasts transfected with viral proteins were reported to directly induce CTL responses in the milieu of lymphoid organs [31].

Based on these considerations, there are several possible mechanisms by which antibody–IL-2 fusion proteins can induce the eradication of disseminated tumor metastases. A possible mechanism is that tumor cells themselves might interact with naive T cells with IL-2 providing the second costimulatory signal for activation of CTL. In this regard, Sprent [32] proposed a model for activation of naive T cells which provide the rationale for this mechanism. Accordingly, high-avidity interaction between peptide–MHC class I complexes and the T-cell receptor promotes strong crosslinking of T-cell receptor–CD3 complexes. This, in turn, leads to strong signaling that stimulates the production of cytokines, such as IL-2 and its receptors; costimulation boosts the T-cell receptor-mediated signal. However, if the signaling intensity is below a certain threshold and the density of peptide–MHC complexes or the level of costimulation is low, the responding T cell expresses IL-2 receptors, but not IL-2. In this case, T cells fail to proliferate, unless exposed to exogenous IL-2. However, this mechanism is unlikely to occur, since naive T cells, in contrast to memory or activated T cells, do not access peripheral tissue [28]. Alternatively, since even low doses of IL-2 could cause a strong infiltration of the skin with CD3+ T cells, the systemic concentration of IL-2 administered by immunocytokines may alter the circulation pattern of T cells [33].

A second possible mechanism accounting for T-cell activation is based on the finding that tumor antigens are being processed by antigen-presenting cells that transfer to the lymph node, where T-cell priming then occurs. It was demonstrated that preactivated macrophages, dendritic cells and granulocytes express receptors for IL-2 and that *in vitro* culture with IL-2 causes functional changes in these cells, such as induction of migration [34–36]. After arriving at the tumor site, these cells may be activated by the antibody-targeted IL-2 to kill the tumor cells and subsequently present the tumor antigens to T cells. The obvious infiltration of monocytic cells within the tumor after administration of the antibody–IL-2 fusion protein supports this hypothesis; however, the requirements for such 'professional' antigen presentation in MHC class I-restricted responses are not yet completely defined [37].

A successful antitumor T-cell response involves induction, recruitment and effector function of T cells. As mentioned above, possible mechanisms involved in IL-2 targeting to the tumor microenvironment may influence the induction phase; however, in contrast to IL-2-producing tumor cells, the therapeutic approach using immunocytokines also addresses the recruit-

ment of CTL and the activation of their effector function. This is quite apparent in view of the effect of antibody–IL-2 fusion proteins on large subcutaneous tumors. Furthermore, the presence of tumor-specific CTL in the spleen of treated mice is more likely to be due to IL-2-mediated expansion of effector cells than to their priming.

The therapeutic efficacy of immunocytokines against micrometastases exhibiting antigen heterogeneity can be explained in two ways, either by induction of an immune response directed against both cell types via the antigen-expressing cell, or by the presence of IL-2 in close proximity to the antigen-negative cells. The latter explanation is supported by the finding that the carboxyl-terminal lysine residue on the heavy chain of ch14.18–IL-2 is accessible to cleavage by plasmin [10]. Therefore, it is possible that in the tumor microenvironment, a portion of the fusion protein is cleaved into ch14.18 and IL-2. Zatloukal *et al.* [24] have demonstrated that an antitumor immune response can be elicited by co-administration of non-modified tumor cells and IL-2-transduced fibroblasts. This observation supports the contention that the antigenic signal and costimulatory signal do not need to be delivered by the same cell.

In conclusion, we demonstrated in this overview the efficacy of antibody-targeted IL-2 for the treatment of established, disseminated melanoma metastases affecting clinically relevant organs. This immunocytokine-induced eradication of tumor metastases is critically dependent on T cells, is MHC class I-restricted and is likely to be due to either priming of naive T cells, the activation of cytotoxic effector cells, or both. It is anticipated that the results of our studies will support and provide the preclinical basis for evaluating the application of antibody–IL-2 fusion proteins in phase I/II trials of patients with disseminated melanoma.

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